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Metagenomic sequencing of activated sludge filamentous bacteria community using the Ion Torrent platform

Congyuan Cao[a], Inchio Lou[a,*], Chen Huang[b], Ming-Yuen Lee[b]

[a]*Faculty of Science and Technology, Department of Civil and Environmental Engineering, University of Macau, Av. Padre Tomás Pereira Taipa, Macau SAR, P.R. China, Tel. +853 6386 9424; email: mb25499@umac.mo (C. Cao), Tel. +853 8397 8469; email: iclou@umac.mo (I. Lou)*
[b]*Institute of Chinese Medical Sciences, University of Macau, Av. Padre Tomás Pereira Taipa, Macau SAR, P.R. China, Tel. +853 6386 4687; email: huangchen11@yahoo.com (C. Huang), Tel. +853 6617 7651; email: simonlee@umac.mo (M.-Y. Lees)*

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ABSTRACT

Filamentous bulking, the most common solids separation problems in wastewater treatment systems, is caused by filamentous bacteria extending outside the flocs. Previous studies using microscopy and conventional molecular techniques, such as fluorescence *in situ* hybridization and real-time qPCR, have difficulty in obtaining the complete profile of filaments. In this study, metagenomic analysis using an Ion Torrent Personal Genome Machine (PGM) platform was adopted to investigate the filamentous bacteria community in Macau wastewater treatment plant that is experiencing filamentous bulking in recent years. The results showed that approximately 571 MB bases of raw data were obtained, acquiring a total of 561,943,741 clean and high-quality bases for further subsequent analysis. *Haliscomenobacter hydrossis* was identified as the dominant filaments that caused bulking. The present study indicated that the total filamentous bacteria could be determined well through PGM sequencing, from which the oligonucleotide probes and primers can be developed and used for targeting the interest species under different operational conditions. In addition, the distributions of Virus, Bacteria, Archaea, Eukaryote domain at read and contig level were determined in this study.

Keywords: Filamentous bulking; Activated sludge; Ion Torrent sequencing; Activated sludge structure

1. Introduction

Filamentous bulking is caused by the excessive growth of various types of filamentous bacteria extending from bioflocs into the bulking solution, resulting in solids inventory problem and high suspended solids concentrations in the effluent [1–4]. Filaments are traditionally identified by microscopy based on morphology and staining characteristics [5]. With the development of molecular techniques, more researches have applied fluorescence *in situ* hybridization and qPCR for identifying and quantifying filaments [6–8]. However, these techniques require precise probes and primers design, which were limited by the trial-and-error approaches and hardly obtain the complete profile of filaments [9].

*Corresponding author.

Currently, sequencing technology is evolving rapidly and shows overwhelmingly superior to the conventional molecular techniques on profiling microorganism community and structure [10–12]. These technologies are undergoing sustained improvement in providing a broad range of applications such as molecular cloning, breeding, finding pathogenic genes, and comparative and evolution. The ideal sequence should be accurate, fast, easy-to-operate, and cheap. 454 pyrosequencing platforms have been reported to reveal the bacterial community composition and diversity of a full-scale integrated fixed-film activated sludge system [13] and the pathogenic bacteria in the sewage treatment plants [14].

The Ion Torrent Personal Genome Machine (PGM) is a new sequencing platform released at the early of 2011 and can perform two hours sequencing run for up to 200 bps read, and the sample preparation time is less than 6 h for eight samples simultaneously [12]. PGM uses semiconductor sequencing technology, which is light independent. The sequence composition is determined by measuring the change of pH due to proton releasing as nucleotides are incorporated into the DNA molecules by the polymerase. By detecting pH change, PGM can recognize whether the nucleotide is added or not. Ion Torrent has released Ion 314, 316, and 318, which are different in the number of wells. The Ion 318 chip can achieve the highest production of 1 GB data in 2 h, with the read length increasing to be greater than 400 bps. It is simple, more cost-effective, and more scalable than any other sequencing technologies. Performance comparison of the three benchtop high-throughput sequencing platforms [11] was made, showing that the Ion Torrent is the lowest price platform, with the cost per base of generating sequence data to be an order of magnitude lower than the 454 GS Junior. Besides, the Ion Torrent PGM is notable for offering three differently priced sequencing-chip reagents, which gives flexibility when designing experiments. It also produced and delivered the fastest throughput and shortest run time. Previous study [15] has demonstrated that Ion Torrent PGM is an effective sequencing platform suitable for studying microbial ecology, including the quality and extent of the output as well as the compatibility with downstream data analysis pipelines and the ability to multiplex microbial community analyses. Thus, it is believed to be a promising method for monitoring filaments in activated sludge samples.

In addition to the filamentous bacteria in the wastewater treatment plants (WWTPs), the community of microorganisms, particularly the pathogenic Bacteria, Archaea, Eukaryote and even Virus in the activated sludge system, is becoming more and more concerns, due to their disease-carrying sources that affect the health of public. Traditionally, indicator microorganisms, such as total coliform and fecal coliform, were used to estimate the contamination level, instead of identifying and quantifying them directly. On the other hand, the Ion Torrent PGM sequence is capable of meeting this task by showing the complete microbial diversity profile in the system.

Macau Peninsula WWTP, occupying around 80% of the total treated volume in the whole area of Macau, is experiencing filamentous bulking in recent years. Macau WWTP is a municipal WWTP that uses the conventional activated sludge system. However, the dominant filaments and the operational conditions of the plants causing bulking are still unknown. In this study, we developed the Ion Torrent PGM sequencing protocols and used them to investigate the community structure of filamentous bacteria and major taxa of the microbial ecology in Macau WWTP, from which the oligonucleotide probes and primers can be developed and used for targeting the interest species under different operational conditions. The originality of this study was the first time to adopt the Ion Torrent PGM sequencing technology for identifying and quantifying the filamentous bacteria community and structure in activated sludge treatment systems.

2. Materials and methods

2.1. Macau Peninsula WWTP and sampling

Macau Peninsula WWTP (113˚33′12′′E in longitude and 22˚12′12′′N in latitude), located in the east part of Macau Peninsula, a humid subtropical climate region, is the largest WWTP in Macau. The plant is a typical municipal wastewater treatment plant, which has the total designed capacity of 356,000 cubic meters per day, and currently treats around 80% of the total volume of wastewater in Macau. It is operated as conventional activated sludge treatment process, that is continuously completely mixed reactor, which is susceptible to filamentous bulking [16–18]. In recent years, the Macau WWTP was reported to experience filamentous bulking with high sludge volume index (SVI) (>150 mL g^{-1}) [6,7] in the summers. The influent concentrations of COD, NH_3-N, TN and TP, and SVIs in 2008–2010 were showed in Figs. 1 and 2, respectively. The SVIs exhibited a periodic pattern each year, with bulking occurred from March to September and non-bulking happened from October to February. The highest SVIs were mostly in June–August with the SVIs up to 800 mL g^{-1}, implying that high temperature favors the filamentous bulking. The bulking percentages (the number of samples with SVI > 150 mL g^{-1}

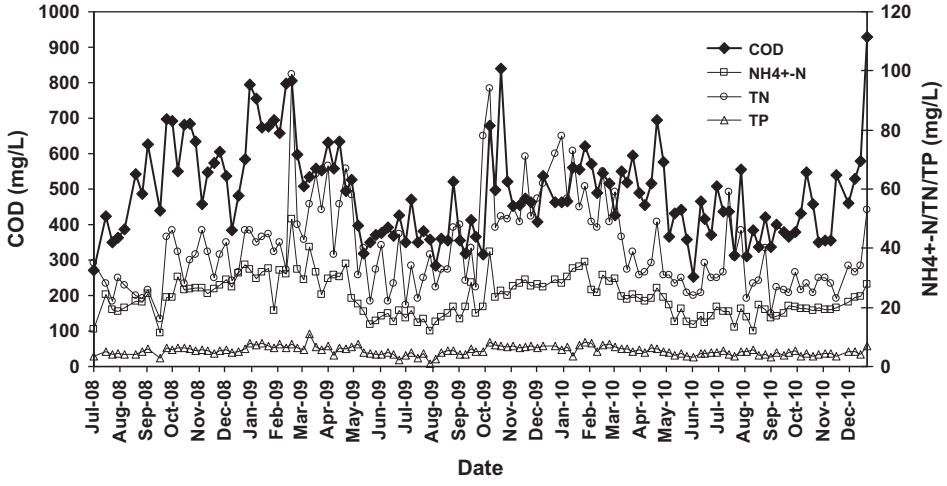

Fig. 1. Change of influent COD, NH_4^+-N, TN and TP over 2008–2010.

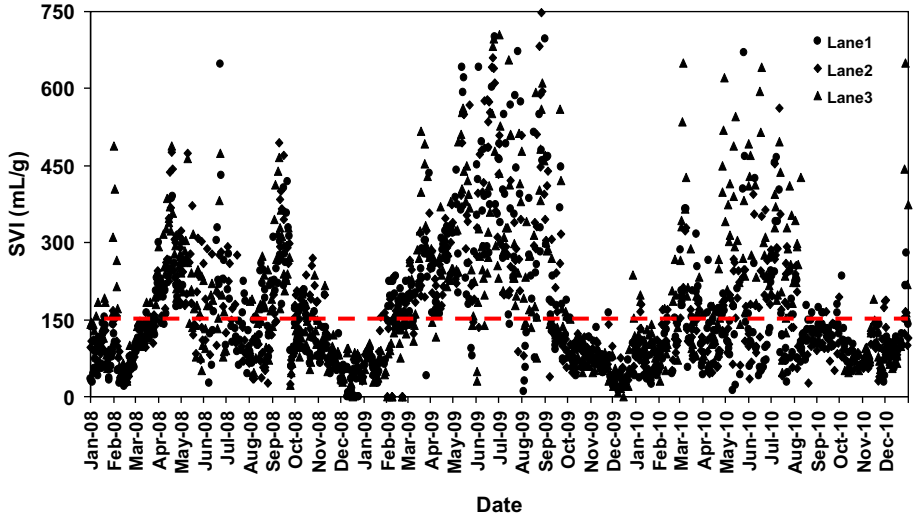

Fig. 2. Change of the SVIs over 2008–2010.

over the total number of samples) were 45.1, 58.5, and 37.3% in 2008, 2009, and 2010, respectively.

In the present study, activated sludge samples were collected on 28 September 2012 from the aeration tank of the Macau Peninsula, with the SVI value of 277 mL g^{-1}, belonging to the medium bulking level, and immediately transferred to the laboratory. The samples were centrifuged at 3,000 rpm for 5 min to remove the supernatant and kept in −20˚C freezer until DNA extraction.

2.2. DNA extraction

The sludge sample was re-suspended at the initial concentration, 200 mL of which was centrifuged at 3,000 rpm for 10 min to obtain the pellet of about 1,000 mg. The DNA from the pellet was extracted using UltraClean® Soil DNA Isolation Kit (Q-Biogene, CA) and then quantified by Qubit 2.0 instrument using the Quant-iT™ dsDNA BR kits (Invitrogen, Carlsbad, CA) prior to storage at −20˚C.

2.3. Identification and quantification by RT-qPCR

Through the Ion Torrent sequencing results, we picked the most read amount of filamentous bacteria, *H. hydrossis*, to identify their abundances (concentrations) by RT-qPCR on 28 September 2012, the same sampling day as by Ion Torrent sequencing.

Table 1
Primers used for qPCR assays

Target	Primer	Sequence (5′–3′)	DNA length	Reference
Haliscomenobacter hydrossis	HHY F HHY R	TTCTGGCGCTGAAGGATGAG GTGTCTCAGTACCCGTGTGG	118 bps	This study

The primer set specific for the 16S rRNA gene of *H. hydrossis* was newly designed in this study, by Invitrogen (Guangzhou, P.R. China). Invitrogen is a global company targeting gene products and services. After designing, primers specificities to the selected sequences in this study were checked in BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of the primer sets used real-time PCR analysis are shown in Table 1. The standard curve for *H. hydrossis* set ranging from 3.0×10^{-2} to 3.0×10^{-6} ng μL^{-1}. The curve was highly linear ($R^2 = 0.98$) in the range tested by the duplicate reactions. The slope of the standard curves for *H. hydrossis* set was −1.35.

2.4. Ion Torrent

2.4.1. Library preparation and sequencing

Barcoded libraries were generated with input 100 ng DNA using the NEBNext™ Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Ipswich, MA) and according to the instruction of DNA fragmentation, adaptor ligation, fragment size selection, and library amplification for a total of eight cycles. The target fragment size around 330 bps was performed with 2% Agarose Gel Cassettes for the Pippin Prep instrument (Sage Science, Beverly, MA). Prior to emulsion PCR, size distribution, and concentration of library were assessed on Bioanalyzer 2100 using the DNA High Sensitivity Kit (Agilent, Santa Clara, CA). Fragment library was adjusted to approximately 26 pM and amplified on the Ion Sphere particles™ (ISPs) by emulsion PCR using Ion OneTouch™ Instrument with Ion OneTouch™ 200 Template Kit (LifeTechnologies, Grand Island, NY), and template-positive ISPs enrichment according to the manufacturer's protocol (Part Number 4472430 Rev. E, 06/2012). The enriched ISPs resulted in over 50% and then sequenced on Ion 316™ chip using the Ion Torrent Personal Genome Machine (PGM™) (Life Technologies, San Francisco, CA) for 130 cycles (520 flows) with Ion PGM™ 200 Sequencing kit (Part Number 4474246 Rev. D, 06/2012).

2.4.2. Data cleaning before analysis

The sequence data were obtained using the Ion Torrent platform, which yielded the raw reads. Before analyzing, the sequence reads were filtered by: (1) removing the reads containing the adapter; (2) removing the sequences shorter than 50 bps; and (3) removing the low-quality sequences (more than 10% of bases below Q20 quality or 5% bases as unknown nucleotides). A total of 3,022,826 reads averaging 185 base pairs were analyzed in this study.

2.4.3. Database and analysis for filamentous bacteria

First of all, the reads were taxonomically classified by BLASTX search against the non-redundant protein sequence (nr) database in the National Center for Biotechnology Information (NCBI) of the National Institute of Health, USA. The E-value of blast search was set to 1×10^{-5}. The blast was carried out as follows: firstly, the blast results were filtered according to two standards: (1) All the reads found the best "hit" according to hit score and identify value and (2) the mapped length must be at least half of the shorter one between query read and subject sequence (this step ensures that the mapped accuracy). Secondly, each read of taxonomic information (kingdom, phylum, class, order, family, genus, and species) was extracted from NCBI taxonomy database according to the hit query gi ID, which has the corresponding taxonomy ID in taxonomy database, this procedure was achieved by some perl scripts. Furthermore, in order to obtain more accurate results, we performed the secondary taxonomic analysis from the assembly contigs aspects. Specifically speaking, the reads were assembled to contigs by Newbler software firstly, and then the contigs were taxonomically classified in the same way as the above-mentioned procedure. The blast results of two methods were compared. Then, the filamentous bacteria [1,19] were selected from the taxonomy database.

2.5. Data processing

Statistical analyses were performed using Microsoft Excel spread sheet.

3. Results and discussion

3.1. Total filamentous bacteria abundances in Macau WWTP

Approximately, 571 MB bases of raw sequence data were obtained before data cleaning. After filtering the low-quality reads and adapter, and removing the too short reads (<50 bps) which may give rise to incorrect blast alignment results, we acquired a total of 561,943,741 clear and high-quality bases for further subsequent analysis. We expected to find out the similarity sequences through BLASTX search against NCBI nr database, which contains the non-redundant proteins sequences of the most species. Then, the detailed taxonomic classification information of each query sequence was obtained by extracting taxonomy information from taxonomy database, according to gi ID of the match sequences.

Table 2 showed the profile of the filamentous bacteria in the sample. There were totally 22 filamentous bacteria sequences belonging to five phyla, *Proteobacteria*, *Flavobacteria*, *Bacteroides* (including CFB group), *Chloroflexi*, and *Planctomycetes*, identified, in which *Bacteroides* is the major phylum occupying 1.32% of the sample. Among all the species of *Bacteroides*, *H. hydrossis* was the most abundant in the sample with around 1.06% of the sample. It has been reported in the previous study [20] that *H. hydrossis* grows well at the temperatures of 8–30˚C with the optimal growth at 25–27˚C, and the presence of *H. hydrossis* is associated with low dissolved oxygen concentrations, low food-to-mass ratio, and nutrient deficiency. These conditions matched the operational conditions in the Macao WWTP, thus favoring the species that was responsible for bulking in the summer. The needle-shaped *H. hydrossis* is the most commonly observed *Bacteroidetes* in industrial and municipal treatment plants, particularly in conventional WWTPs without nitrogen removal (carbon removal with/without nitrification, but no denitrification) treating primarily municipal wastewater [21]. These plants are usually low/medium loaded with medium/long sludge age [20]. Other filaments including Type 0411 (*Runella*), *Sphaerotilus natans Leptothrix*, and Type 1863 (*Chryseobacterium*) were also observed at high

Table 2
The profile of filamentous bacteria in the activated sludge sample of Macau Peninsula WWTP

Types	Phylum	Classifier by the reference sequence	Accession No. in NCBI	Abundance in the sample	
				Reads	In (%)
Haliscomenobacter hydrossis	*Bacteroidetes*	*Haliscomenobacter hydrossis*	NR_042316.1	31,945	1.06
	Bacteroidetes	*Sphingobacterium spiritivorum*	NZ_GG668649.1	465	0.02
	Bacteroidetes	*Prevotella dentalis*	NZ_GL982496.1	333	0.01
	Bacteroidetes	*Prevotella buccae*	NR_044631.1	117	0.00
Type 0411	*Bacteroidetes*	*Runella*	X85209.1	3,941	0.13
Type 1863	*Bacteroidetes*	*Chryseobacterium*	X95304.1	2,919	0.10
Type 0092	*Chloroflexi* CFB	*Flavobacterium*	X85210.1	1,887	0.06
Type 1851	*"Kouleothrix aurantiaca"*	*Chloroflexus aggregans*	D32255	63	0.00
		Chloroflexus aurantiacus	D38365	95	0.00
		Roseiflexus castenholzii	AB041226	51	0.00
"Nostocoida limicola" III	*Planctomycetes*	*Singulisphaera*	JN367232.1	911	0.03
	Planctomycetes	*Singulisphaera acidiphila*	NR_042662.1	911	0.03
	Planctomycetes	*Isosphaera pallida*	X64372	363	0.01
Sphaerotilus natans	*Proteobacteria*	*Leptothrix*	GU591793.1	3,686	0.12
	Proteobacteria	*Acidovorax delafieldii*	AF078764	744	0.02
	Proteobacteria	*Acinetobacter junii*	HE798195.1	442	0.01
	Proteobacteria	*Acinetobacter johnsonii*	X81663	353	0.01
Beggiatoa	*Proteobacteria*	*Beggiatoa*	NR_041726.1	203	0.00
Type 021 N	*Proteobacteria*	*Thiothrix*	L79965.1	1	0.00
	Chloroflexi	*Anaerolinea thermophila*	AB046413	991	0.03
	Chloroflexi	*Chloroflexus aurantiacus*	NR_043411.1	95	0.00
	Chloroflexi	*Herpetosiphon aurantiacus*	M34117	308	0.01

frequencies and considerable abundances in the samples (Table 2). The abundance of the total filamentous bulking was estimated as about 1.65% of the sludge, which was less than 2–6% estimated in the 12 of 14 sewage treatment using the pyrosequencing technique in the previous study [9]. This is because that the previous study was based on detecting BFB (bulking and foaming bacteria), while our study only targeting the bulking-causative bacteria.

Thiothrix spp., another type of filament that is hardly detected by microscopy based on morphology and staining characteristics, can also be quantified by the Ion Torrent PGM sequencing with 0.01% of sludge. However, other prevalent bulking-causative species including Type 0041, Type 0675, Type 0803, Type 1701 [1], *M. parvicella* [22], Type 0914 [23], and *Leucothrix mucor* [24] were not observed in this study. The different results between surveying

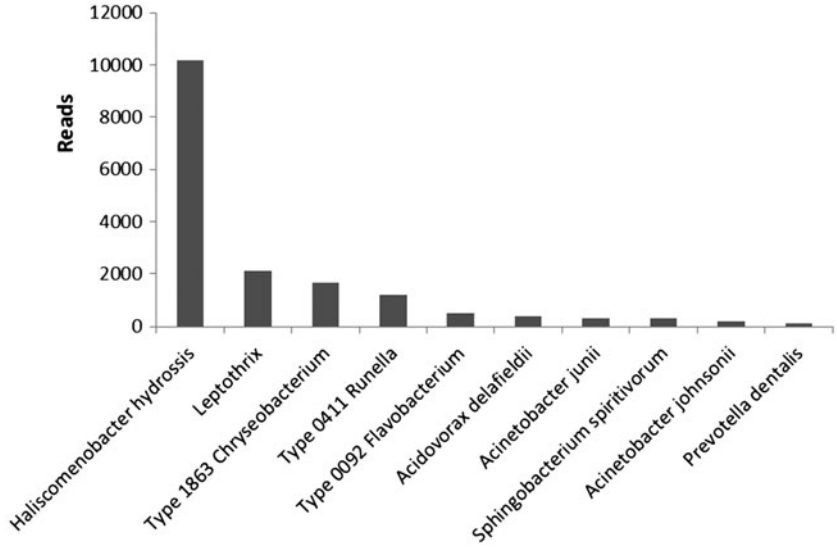

Fig. 3. Reads of the various filamentous bacteria groups.

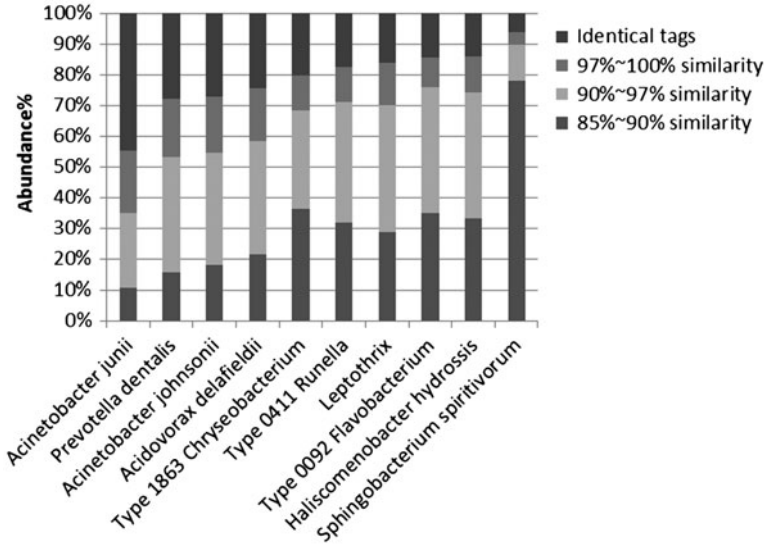

Fig. 4. Abundance of the various filamentous bacteria groups at each cutoff value of similarity. Only the filamentous bacteria groups with over 20 bits were calculated. The left groups had more similar pyrotags, while the right groups had more relatively remote tags.

non-bulking and bulking sludge suggested that some groups of species may be opportunistic filamentous bacteria, which stick outside from flocs and cause high SVI only under certain operational conditions, and other groups such as Type 021 N may be more virulent because once emerge, it always overgrow and cause bulking.

Our results showed that using the Ion Torrent PGM sequencing, the dominant species presented at high frequencies and abundances in the samples would be considered as responsible bulking-causing filaments in the Macau WWTP.

To further evaluate the taxonomic assignment of the ion tags, the abundances of the ion tags at various cutoff values (99, 97, 90, and 85%) for various filamentous bacteria groups (only >20 hit identical tags in all samples were considered) were calculated, as shown in Figs. 3 and 4. The results showed that the dominant filamentous bacteria are *Haliscomenobacter*, Type 0411, *Leptothrix*, and Type 1863 in Macau Peninsula WWTP. Ninety seven percentage cutoff value is the general threshold value for the bacterial species level [25]. The filamentous bacteria in Macau WWTP are 1% of total bacteria. Filamentous bacteria profile at 97% similarity cutoff value in Macau WWTP was shown in Fig. 3.

The 22 hit filamentous bacteria groups were ranked by both their similarity and abundances, as listed in Table 2. The groups with high abundances usually had high similarity. It noted that from Fig. 4, *Sphingobacterium spiritivorum* has much lower similar ion tags, implying that (1) there are certain loosely-related groups in the sample that are not belonging to this filamentous bacteria type and (2) there is higher variation of whole gene within the same filamentous group.

In the further study, we can choose the dominant filamentous bacteria to determine the relationship among their abundances and bulking levels represented as SVI values and to relate their amount to the operational conditions. Thus, rich information from the Ion Torrent PGM deep sequencing could be helpful in developing oligonucleotide probes and primers for specific species under different operational conditions in lab-scale study.

3.2. Results of RT-qPCR

Using RT-qPCR approach, the concentrations of *H. hydrossis* and *Thiothrix* spp. were quantified as 4.12×10^9 copies g^{-1} sludge and 5.31×10^7 copies g^{-1} sludge, respectively, that is, there is around 100 times *H. hydrossis* copies more detected than *Thiothrix* spp.'s by qPCR, while about 30,000 times *H. hydrossis* gene read more identified than *Thiothrix* spp.'s (only 1 gene read) by metagenomic sequencing (31,945 gene reads). The large difference of the corresponding ratios quantified by qPCR and metagenomic sequencing is probably due to the different principles and sensitivities of both methods, in which metagenomic analysis performs assembly, annotation and database comparison while qPCR directly targeting the specific 16S DNA regions. However, further study is required to make comparison in the future.

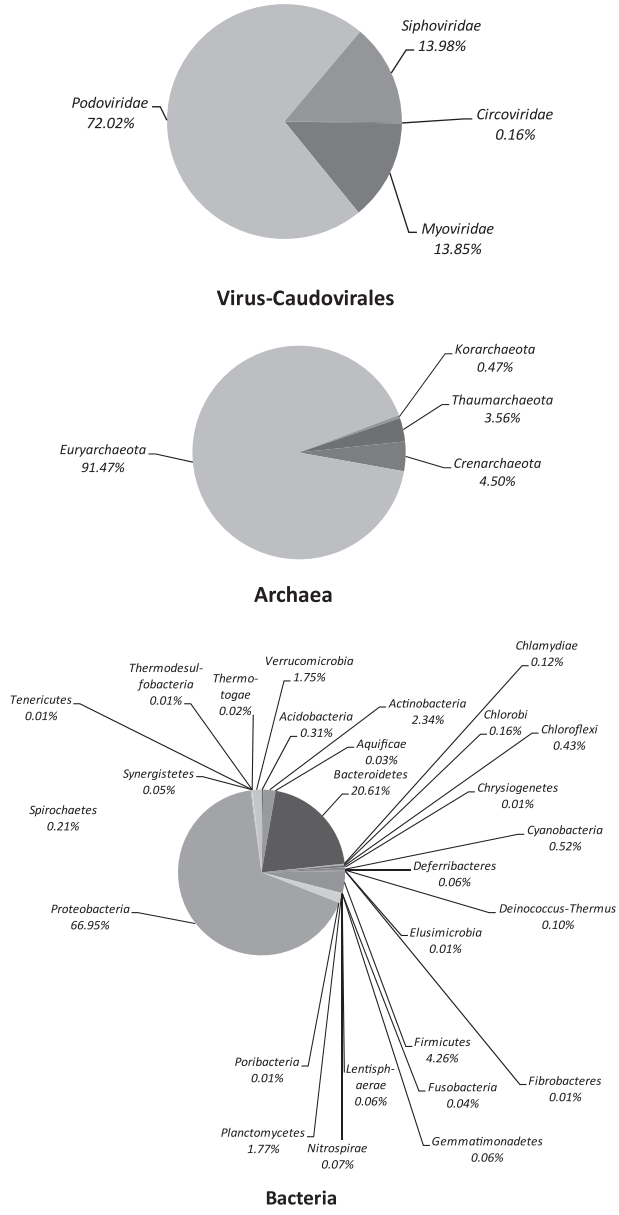

Fig. 5. Distribution of Virus, Archaea, and Bacteria domain as determined by taxonomic identification at read level.

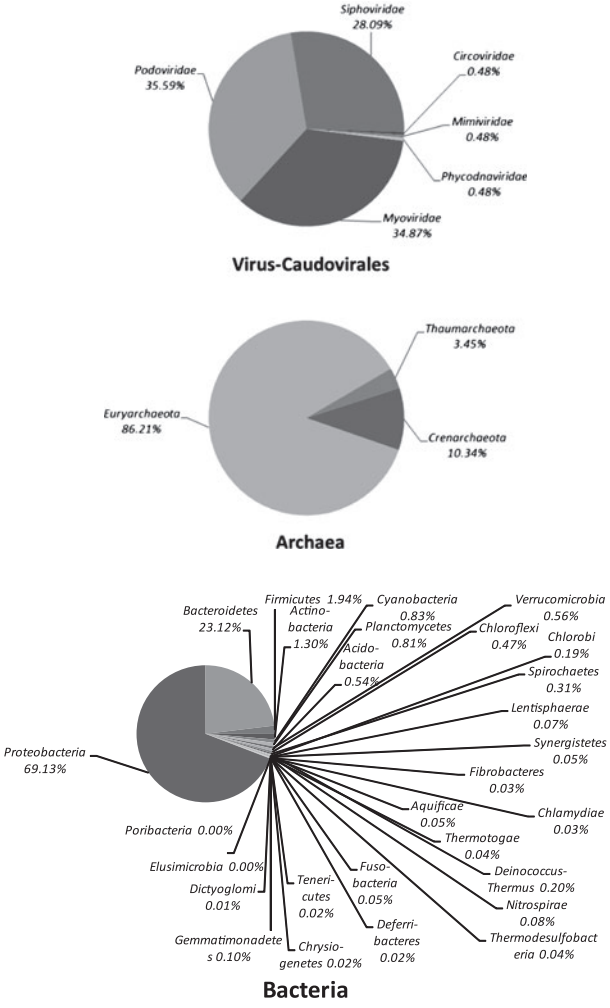

Fig. 6. Distribution of Virus, Archaea, and Bacteria domain as determined by taxonomic identification at contig level.

3.3. Microbial community structures in Macau WWTP

In this study, about 27% (823,672 reads) of data retained after the blast filter, according to the above-mentioned criteria, and 776,100 reads were annotated to the species taxonomy information. It can be divided into three categories: Archaea, Bacteria and Virus and the details were shown in Figs. 5 (by reads) and 6 (by contigs). It is clear that the distribution of community generated from analysis of contigs is very similar to that of reads, except that the corresponding ratios of the subsets. Both results of taxonomic analysis on contig and read sequences show a high level of consistency. Compared with the qPCR, metagenomic sequencing approach not only provides more accurate information of the diversity of microbial community, but also identifies the virus compositions in wastewater treatment sample, suggesting that this powerful tool can bring new vision and perspectives to future research.

Compared with other sequence platforms, the Ion Torrent PGM provides useful insights into the microbial ecology, which is poised to make a decisive impact on public health microbiology in the future, particularly in understanding microbial community dynamics and their impact on the functional potential of environmental microbial system, which allows a fundamental basis to study the microbial diversity and functionality in natural and engineered microbial ecosystems.

4. Conclusions

Our study showed that the total filamentous bacteria could be identified and quantified through the high-throughput Ion Torrent PGM sequencing. It showed that *H. hydrossis*, Type 0411, *Sphaerotilus natans*, and Type 1863 were identified as the dominant species that would be considered as the responsible bulking-causing filaments in the Macau WWTP, which occupied 1.06, 0.13, 0.12, and 0.10%, respectively, of the total reads of sludge sample. Based on our sequencing results, the corresponding oligonucleotide probes and primers can be developed and used for targeting the interest species under different operational conditions, thus, the relationship among their abundances and bulking levels represented as SVI values can be determined.

In addition, the distributions of Virus, Bacteria, Archaea, and Eukaryote domains at read and contig level were determined in this study, indicating the Ion Torrent PGM sequencing provides more accurate information of the diversity of microbial community and the virus community, which brings new vision to future research in the environmental science and health.

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